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Published in:
 ChemBioChem

DOI:
 [10.1002/cbic.201000517](https://doi.org/10.1002/cbic.201000517)

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Document Version
 Publisher's PDF, also known as Version of record

Publication date:
 2010

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Citation for published version (APA):

Oltra, N. S., Bos, J., & Roelfes, G. (2010). Control over Enzymatic Activity by DNA-Directed Split Enzyme Reassembly. *ChemBioChem*, 11(16), 2255-2258. <https://doi.org/10.1002/cbic.201000517>

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Supporting Information

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Control over Enzymatic Activity by DNA-Directed Split Enzyme Reassembly

Núria Sancho Oltra, Jeffrey Bos, and Gerard Roelfes^{*[a]}

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General remarks

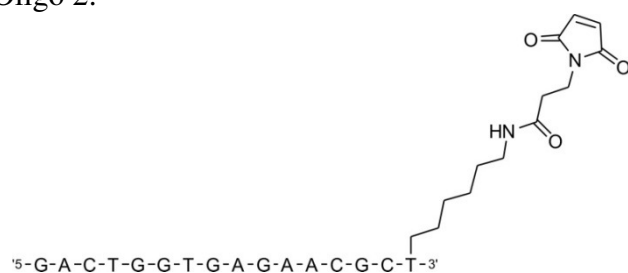
3-maleimidopropionic acid *N*-hydroxysuccinimide ester was purchased from Alfa Aesar. Amino modified oligonucleotides were purchased from Biotex Berlin. *E. coli* strains XL1-Blue and B121 (DE3) (Stratagene) were used for routine cloning and protein production, respectively. PCR reactions were carried out using a Techne TC-312 apparatus. DNA sequencing was carried out by GATC-Biotech (Berlin, Germany). Primers were synthesized by Isogen Life Science (De Meern, the Netherlands). Restriction endonucleases and plasmid pTWIN1 were purchased from New England Biolabs. T4 DNA ligase, DNA Gel Extraction Kit and Plasmid Purifying Kit were purchased from Roche. *Pfu Turbo* polymerase was purchased from Stratagene. Plasmid pQE-30Xa was purchased from Qiagen. Plasmid pET-17b was purchased from Novagen. Commercially available plasmid pQE-16 was kindly provided by Prof. Dr. S.W. Michnick (University Montreal, Canada). DNA manipulations were done by standard procedures.^[1]

Ni-NTA agarose was purchased from Qiagen. HiTrap QFF column was purchased from GE Healthcare. Reversed phase-HPLC analysis were performed on a Shimadzu LC-10AD VP, Waters Xterra MS C18 column (3.0 x 150 mm, particle size 3.5 μm) using a gradient of CH_3CN /triethylammonium acetate (TEAA) buffer 50 mM pH 7; gradient: 05:95 0 to 10 min, to 35:65 at 60 min, to 70/30 at 65 min. Flow: 0.5 mL/min. Centrifugation was done using a Beckman Coulter Avanti J-E centrifuge. Size exclusion chromatography was done using a Superdex 75 HR 10/30 column from Pharmacia. MALDI-TOF measurements were done on a Voyager-DE Pro apparatus. (Matrix: 20 μL of a solution of 2,4,6-trihydroxyacetophenone 0.5 M in ethanol + 10 μL of a solution of ammonium citrate dibasic 0.1 M in Milli Q water + 2 μL sample solution in Milli Q water). Concentration determinations were done using a Nanodrop ND-1000 from Thermo Scientific using the calculated extinction coefficients $\epsilon_{260\text{ nm}} = 163840\text{ M}^{-1}\text{cm}^{-1}$ for oligo 1 and $\epsilon_{260\text{ nm}} = 179210\text{ M}^{-1}\text{cm}^{-1}$ for oligo 2. The same values were used for the corresponding mDHFR-oligo conjugates, since the absorption of the protein fragments at 260 nm is negligible compared to the oligonucleotide absorption. UV/Vis measurements were recorded on a JASCO V-560/V-570 UV/Vis Spectrometer at 25°C. mDHFR assays were conducted in quartz cuvettes with a 1 cm path length. Äkta Purifier 900 (Amersham Biosciences) was used for Fast Protein Liquid Chromatography (FPLC).

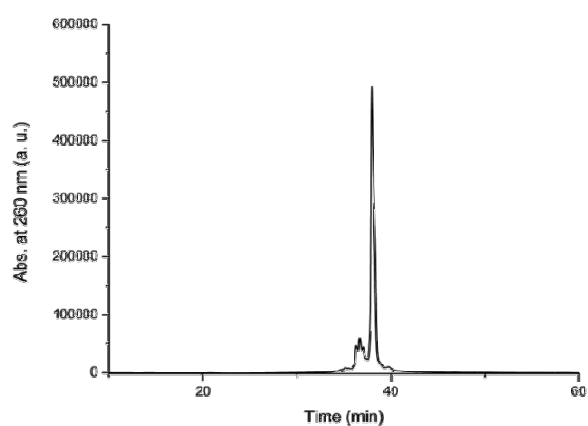
Synthesis of maleimido-DNA conjugates, representative procedure:

182 μL of a stock solution of amino modified-oligonucleotide (200 μM in H_2O) was mixed with 236 μL of Phosphate buffer (200 mM, pH 7.2). To this solution, 60 μL of a stock solution of 3-maleimidopropionic acid *N*-hydroxysuccinimide ester (30 mg/mL in *N,N*-dimethylformamide) was added. The mixture was gently mixed and allowed stand for 2 h. Longer incubation times resulted in hydrolysis of the maleimide ring. The conjugate was purified by size exclusion chromatography (SephadexTM G-25 DNA Grade, TEAA 50 mM pH 7) and lyophilized. The products were analyzed by reversed phase-HPLC and MALDI-TOF.

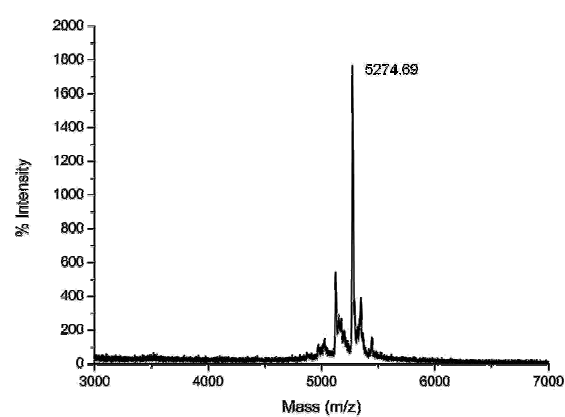
Oligo 2:



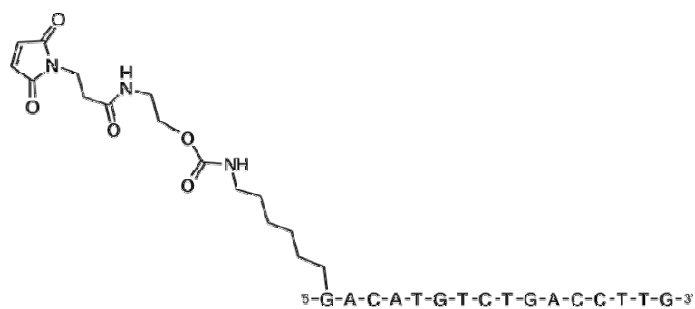
rp-HPLC:



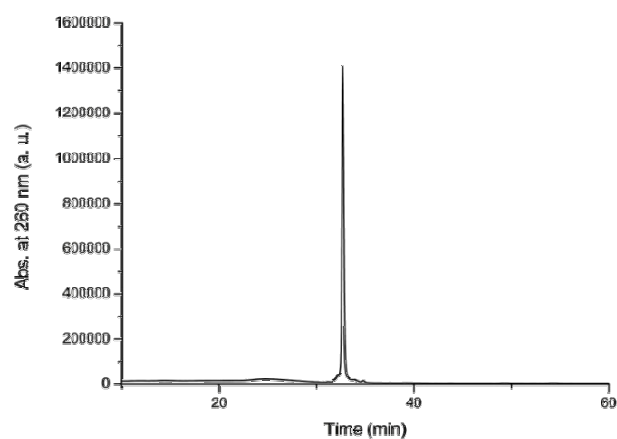
MALDI-TOF (calcd (m/z) = 5276)



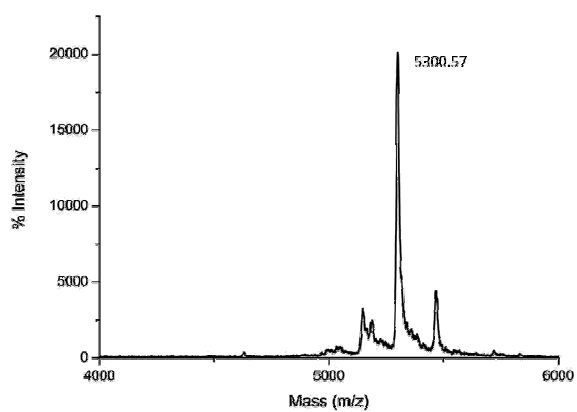
Oligo 1:



rp-HPLC:



MALDI-TOF (calcd (m/z) = 5290)



Construction of expression plasmid pTWINXa:

The pTWINXa plasmid was derived from commercially available plasmids pTWIN1 and pQE-30Xa.

The intein part of the pTWIN1 vector was substituted by the multiple cloning site (MCS) of pQE-30Xa, including the 6x His-tag and factor Xa recognition site. The MCS including the 6x His-tag and factor Xa recognition site was amplified by polymerase chain reaction (PCR) using pQE-30Xa as template. PCR primers were as following; primer 1: 5'-TACTACCATATGAGAGGATCGCATC-3' (including *NdeI* restriction site, underlined), primer 2: 5'-GCTCAGCTAATTAAGCTT-3'. PCR cycles were as following: initial denaturation at 94 °C for 5 min. Denaturation at 94 °C for 1 min, annealing at 40 °C for 45 sec., extension at 72 °C for 25 sec, for 30 cycles. Final extension at 72 °C for 5 min. The obtained PCR product was digested with *NdeI* and *PstI*, and inserted between the same sites of the expression vector pTWIN1.

Construction of expression plasmid pTWINXa-mDHFR:

The mDHFR gene was amplified by PCR using pQE-16 as template. PCR primers were as following; primer 1: 5'-GTTCGACCATTGAACTCGAT-3', primer 2: 5'-TACTACCTGCAGTTAATCTTCTCTCGTAGACTTCAA-3' (including *PstI* restriction site, underlined). PCR cycles were as following: initial denaturation at 94 °C for 5 min. Denaturation at 94 °C for 1 min, annealing at 42 °C for 45 sec., extension at 72 °C for 4 min, for 30 cycles. Final extension at 72 °C for 5 min. The obtained PCR product was digested with *PstI* and pTWINXa was digested with *StuI* (blunt-end cutter) and *PstI* and both were ligated together.

Construction of expression plasmid pTWINXa-N_{term}-mDHFR:

The N_{term}-mDHFR gene was amplified by PCR using pQE-16 as template. PCR primers were as following; primer 1: 5'-GTTCGACCATTGAACTCGAT-3', primer 2: 5'-ACTACCTGCAGTTAGCACAATTCCGGTTGTTCAATAA-3' (including DNA sequence for the incorporation of a cysteine, underlined). PCR cycles were as following: initial denaturation at 94 °C for 5 min. Denaturation at 94 °C for 1 min, annealing at 50 °C for 45 sec., extension at 72 °C for 25 sec., for 15 cycles. Followed by denaturation at 94 °C for 1 min, annealing at 48 °C for 45 sec., extension at 72 °C for 25 sec., for 15 cycles. Final extension at 72 °C for 5 min. The obtained PCR product was digested with *PstI* and pTWINXa was digested with *StuI* (blunt-end cutter) and *PstI* and both were ligated together.

Construction of expression plasmid pTWINXa-C_{term}-mDHFR (1):

The C_{term}-mDHFR (1) gene was amplified by PCR using pQE-16 as template. PCR primers were as following; primer 1: 5'-TACTACATATGGGCTGCGCAAGTAAAGTAGACA-3' (including *NdeI* restriction site, underlined), primer 2: 5'-TACTACTGCAGCTCAGCTAATT AAGCTT-3' (including *PstI* restriction site, underlined). PCR cycles were as following: initial denaturation at 94 °C for 5 min. Denaturation at 94 °C for 1 min, annealing at 42 °C for 45 sec., extension at 72 °C for 50 sec., for 30 cycles. Final extension at 72 °C for 5 min. The obtained PCR product was digested with *NdeI* and *PstI*, and inserted between the same sites of the expression vector pTWIN1Xa.

Construction of expression plasmid pTWINXa-C_{term}-mDHFR (2):

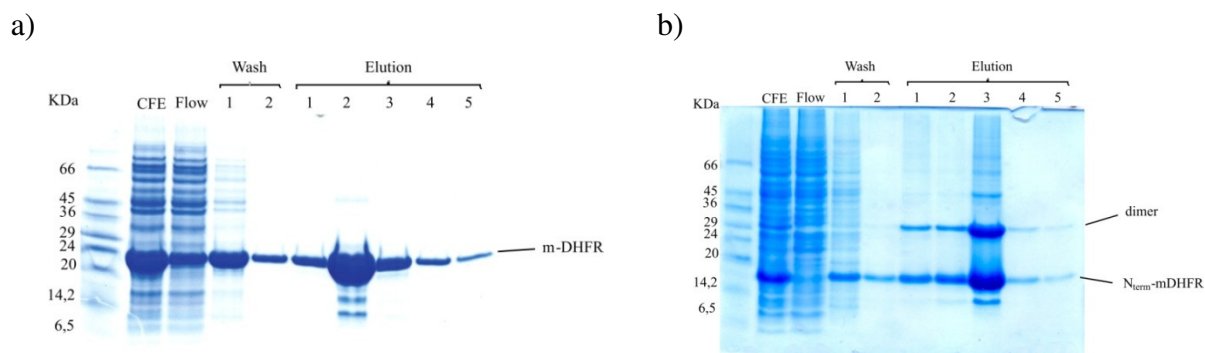
The C_{term}-mDHFR (2) gene was amplified by PCR using pTWINXa-C_{term}-mDHFR (1) as template, introducing a glycine/serine linker. PCR primers were as following: primer 1: 5'-TACTACCATATGGGCTGCGGCGGCAGTGGCGGCGCAAGTAAAGTAGACATGGTTTGG-3' (including *NdeI* restriction site, underlined; Glycine/serine linker in *Italic*), primer 2: 5'-GTAGTACTGCAGTTAGTGATGGTGATGGTATGAG-3' (including *PstI* restriction site, underlined). PCR cycles were as following: initial denaturation at 94 °C for 5 min. Denaturation at 94 °C for 1 min, annealing at 45°C for 45 sec., extension at 72°C for 90 sec., for 30 cycles. Final extension at 72 °C for 5 min. The obtained PCR product was digested with *NdeI* and *PstI*, and inserted between the same sites of the expression vector pET17b.

Expression and purification of mDHFR constructs:

Protein expression plasmids of the individual N_{term}-mDHFR, C_{term}-mDHFR and mDHFR constructs were transformed into *E. coli* BL21 (DE3) and a single colony was inoculated into a starter culture of 5 mL of fresh LB medium containing 100 µg/mL of ampicillin. 1 mL of starter culture was used to inoculate 200 mL of fresh LB medium containing 100 µg/mL of ampicillin. When the culture reached the mid-log phase (optical density at 600 nm around 0.6–0.8) isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to induce the expression of target protein at final concentration of 1 mM. Expressions were done at 37 °C for N_{term}-mDHFR and mDHFR and at 30 °C for C_{term}-mDHFR overnight. Cells were harvested by centrifugation (6000 rpm, JA-10, 25 min, 4 °C), followed by resuspension in 4 mL of 100 mM NaH₂PO₄, pH 8.0, 100 mM Tris-HCl, 8 M urea. The lysate was centrifuged (10000 rpm, JA-17, 25 min, r.t.). The supernatant was equilibrated with 2 mL of pre-equilibrated slurry of Ni-NTA (50% Ni-NTA in 20% Ethanol) for 1 h (mixed at 200 rpm on a rotary shaker) at room temperature. The column was washed with 2 fractions of 4 mL of 100 mM NaH₂PO₄, pH 6.3, 100 mM Tris-HCl, 8 M urea, 10 mM imidazole, subsequently, eluted with 5 fractions of 0.5 mL of 100 mM NaH₂PO₄, pH 4.0, 100 mM Tris-HCl, 8 M urea. Fractions were analyzed on a 12% polyacrylamide SDS-Tris Tricine gel followed by Coomassie staining. The concentration of the protein fragments was determined by using the following calculated extinction coefficients: N_{term}-mDHFR $\epsilon_{280} = 12490 \text{ M}^{-1} \cdot \text{cm}^{-1}$, C_{term}-mDHFR $\epsilon_{280} = 12950 \text{ M}^{-1} \cdot \text{cm}^{-1}$. The literature^[2] value for mDHFR is $\epsilon_{280} = 74600 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Calculations were done by ProtParam on the ExPASy server).^[3]

Expression yields were: 11 mg/L for N_{term}-mDHFR, 29 mg/L for C_{term}-mDHFR (1) and 5 mg/L for C_{term}-mDHFR (2)

MS (ESI) N_{term}-mDHFR 14345 Da (calcd 14341 Da); C_{term}-mDHFR (1) 10694 Da (calcd 10824 Da, calcd [M-Met] 10692 Da), C_{term}-mDHFR (2) 11009 Da (calcd 11140 Da, calcd [M-Met] 11008 Da)



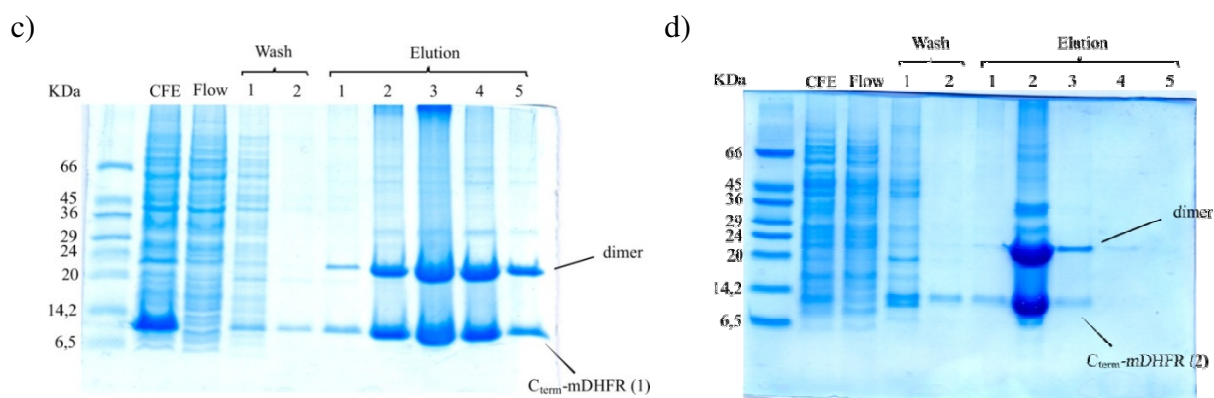


Figure S1. 12% polyacrylamide SDS-Tris Tricine gels of the purification by Ni-NTA of a) mDHFR, b) N_{term}-mDHFR, c) C_{term}-mDHFR (1) and d) C_{term}-mDHFR (2).

General procedure for the synthesis of protein-DNA conjugates:

A volume of the mDHFR fragment solution corresponding to 93 nmol of protein was brought to pH 8 using NaOH 6 M solution and incubated with dithiothreitol (DTT), 0.1 M solution for 1 hour at room temperature. To this solution, 1 mL of 25% trifluoroacetic acid (TFA) was added and the mixture was incubated on ice for 1 h. The mixture was centrifuged at maximum speed for 5 min and the pellet was washed with water several times. The pellet was redissolved in (8 M urea) 100 mM CHES, pH 9.2 to a final concentration of 80 μ M. The solution was immediately transferred to the freeze-dried maleimide modified oligonucleotide (1:1 ratio of functionalized oligonucleotide/mDHFR fragment). The mixture was rotated by a rotator (100 rpm) at 40 °C for 16 h. The extent of coupling was analyzed on a 12% polyacrylamide SDS-Tris Tricine gel, following Coumassie staining.

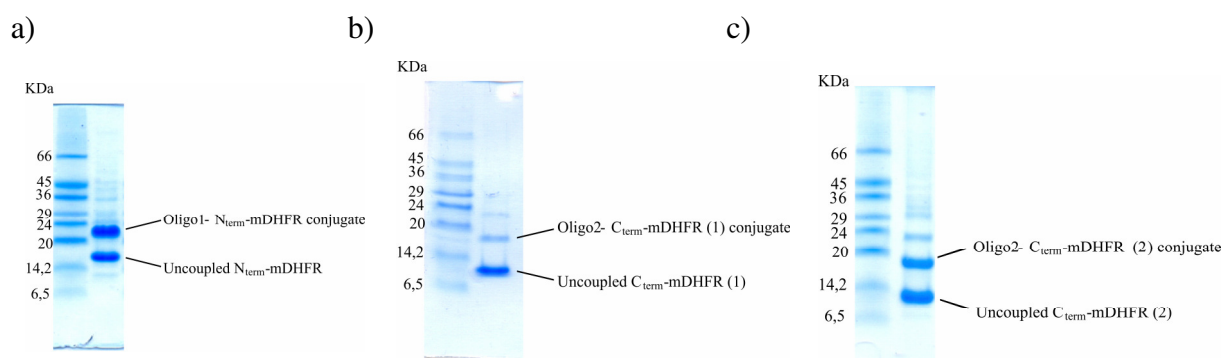
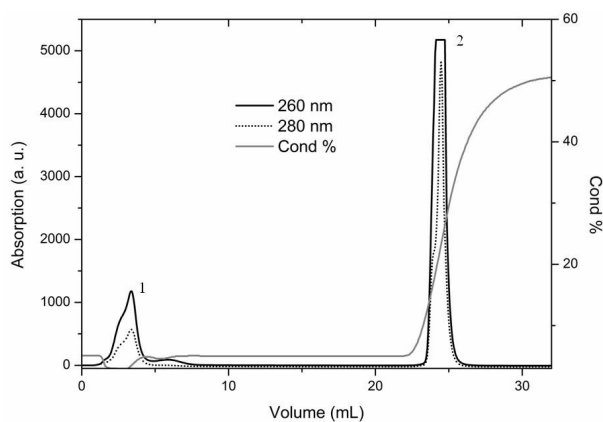


Figure S2. 12% polyacrylamide SDS-Tris Tricine gels of the reaction mixture of a) Oligo 1 + N_{term}-mDHFR, b) Oligo 2 + C_{term}-mDHFR (1) and c) Oligo 2 + C_{term}-mDHFR (2)

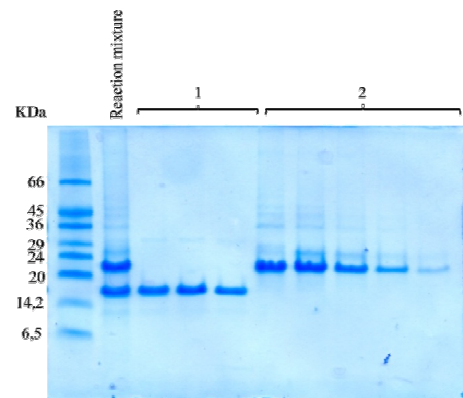
General procedure for the purification of the protein-oligonucleotide conjugates:

- Purification from uncoupled mDHFR fragments: The conjugates were purified by anion-exchange chromatography on a HiTrap QFF column by a gradient of NaCl concentration from 0 to 1 M in 5 min with a flow of 0.5 mL \cdot min⁻¹ in a 100 mM Tris-HCl, pH 7.5, 8 M urea buffer. Elution fractions were analyzed by SDS-PAGE.

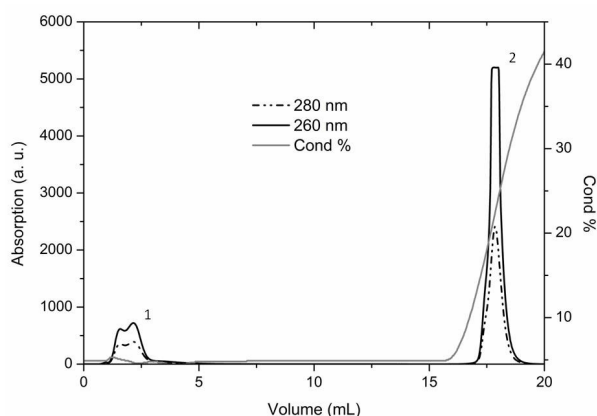
a)



b)

1 – Elution fractions uncoupled N_{term} -mDHFR2 – Elution fractions Oligo1- N_{term} -mDHFR conjugate

c)



d)

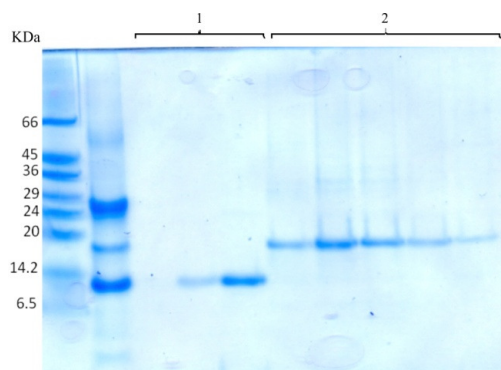
1 – Elution fractions uncoupled C_{term} -mDHFR (2)2 – Elution fractions Oligo2- C_{term} -mDHFR (2) conjugate

Figure S3. Purification of oligo-protein conjugates from uncoupled protein via anion-exchange chromatography. a) UV traces of the elution fractions for the coupling of Oligo1 with N_{term} -mDHFR, b) 12% polyacrylamide SDS-Tris Tricine gels of eluted fractions for the coupling of Oligo1 with N_{term} -mDHFR, c) UV traces of the elution fractions for the coupling of Oligo2 with C_{term} -mDHFR (2), d) 12% polyacrylamide SDS-Tris Tricine gels of eluted fractions for the coupling of Oligo2 with C_{term} -mDHFR (2).

- Purification from uncoupled oligonucleotide: The conjugates were purified by Ni-NTA agarose column. The pooled fractions from the anion exchange column containing the conjugate were equilibrated with 100 μL of slurry Ni-NTA (50% Ni-NTA in 20% Ethanol) for 0.5 h. The column was pre-equilibrated with 100 mM NaH_2PO_4 , pH 8.0, 100 mM Tris-HCl, 8 M urea. Subsequently, the column was washed with 2 fractions of 500 μL of 100 mM NaH_2PO_4 , pH 8.0, 100 mM Tris-HCl, 8 M urea and eluted with 8 fractions of 50 μL of 100 mM NaH_2PO_4 , pH 4.0, 100 mM Tris-HCl, 8 M urea.

Characterization of protein-oligonucleotide conjugates by size exclusion chromatography:

The conjugates were analyzed by analytical size exclusion chromatography using a Superdex 75 HR 10/30 column, pre-equilibrated with 100 mM Tris-HCl, pH 7.5, 8 M urea buffer. The elution volume of the conjugates is at 9 mL.

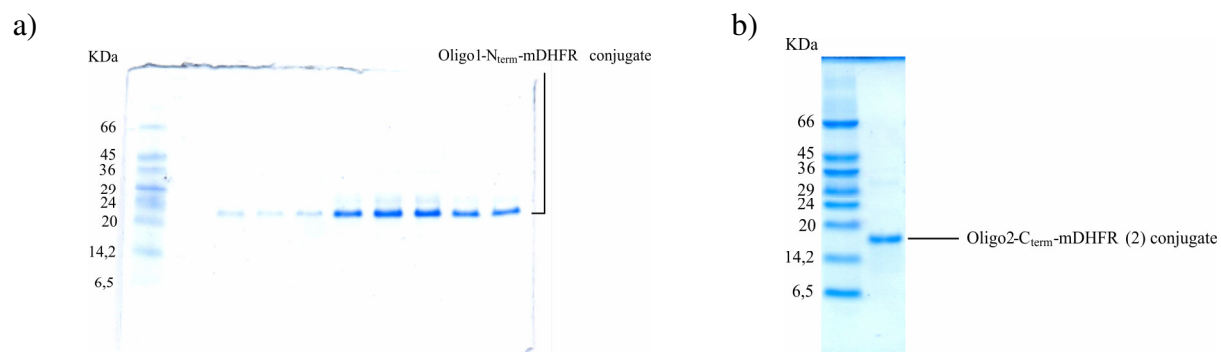


Figure S4. 12% polyacrylamide SDS-Tris Tricine gels of pure a) Oligo1-N_{term}-mDHFR and b) Oligo2-C_{term}-mDHFR (2) conjugates after Ni-NTA column.

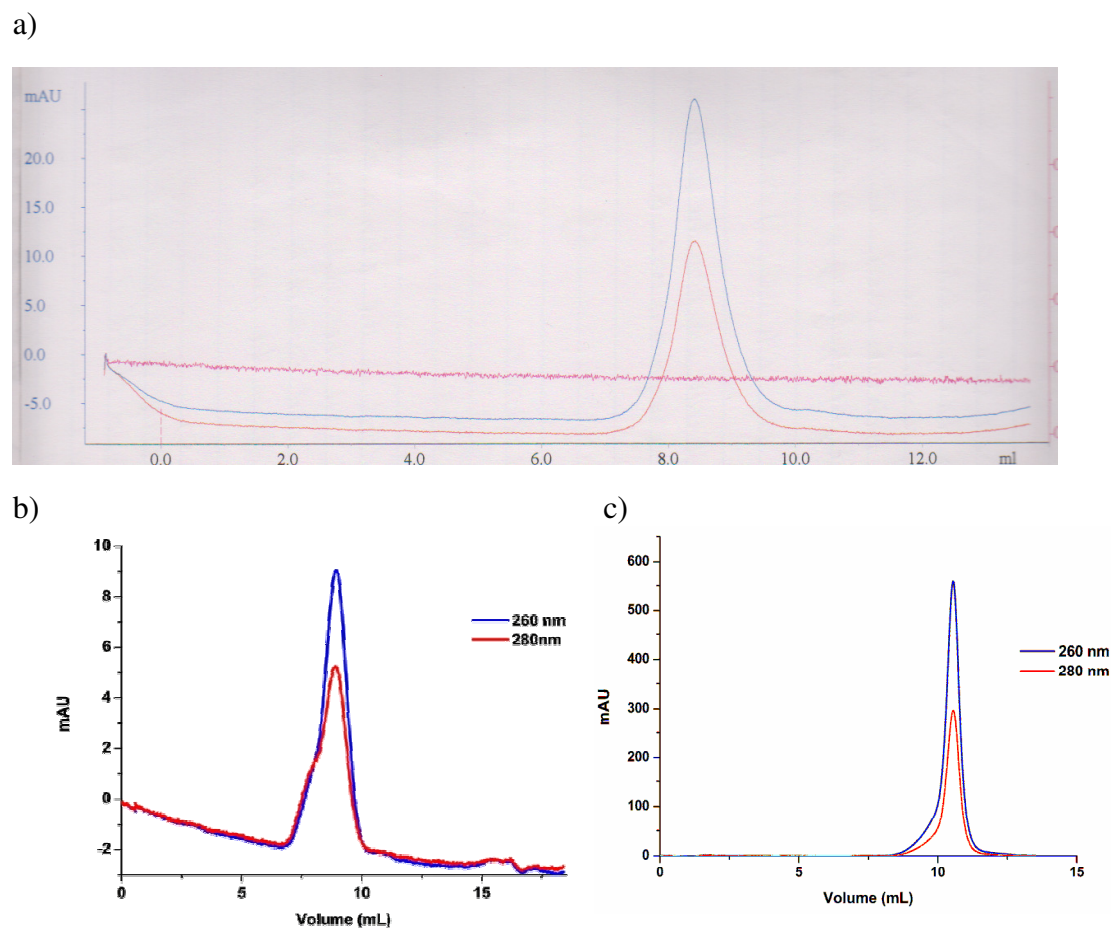


Figure S5. Traces of analytical size exclusion chromatography (UV-Vis detection) of a) Oligo1-N_{term}-mDHFR, b) Oligo2-C_{term}-mDHFR (2) conjugates and c) Oligo 2.

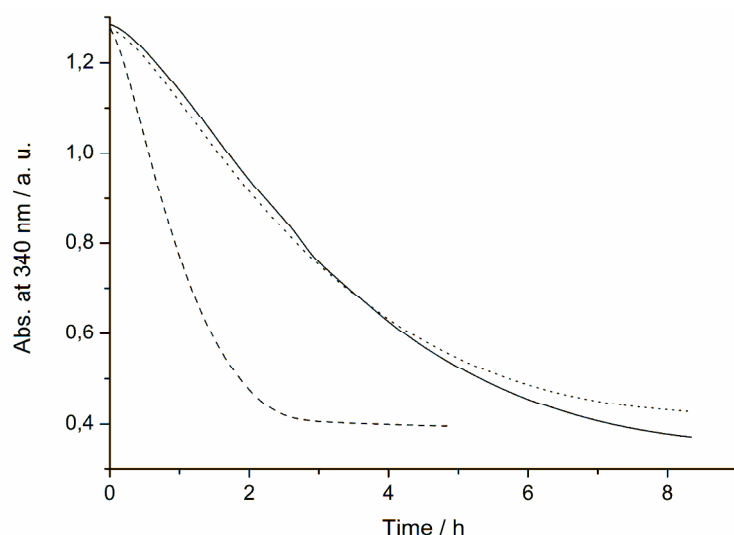


Figure S6. Kinetic curves for the consumption of NADPH in the reduction of dihydrofolate to tetrahydrofolate for equimolar fully complementary systems with preincubation of the system in the reaction buffer (—), preincubation in the reaction buffer containing dihydrofolate (...) and without preincubation (---).

Template sequences: (mutations appear in bold and are underlined, central hyphen indicates the two parts of the template complementary to each of the 16-mer-protein conjugates)

0 mutations: 5'- CAAGGTCAGACATGTC-AGCGTTCTCACCAGTC - 3'

1 mutation: 5'- CAAGGTCAGACATGTT**T**-AGCGTTCTCACCAGTC - 3'

3 mutations: 5'- CAAGGTCAGACATGTT**T**-AGC**A**TTCTCA**T**CAGTC - 3'

5 mutations: 5'- CAAGGTCAGAC**G****T****A****T**-AGC**A**TTCTCA**T**CAGTC - 3'

Protein sequences:

Full length m-DHFR

MRGSHHHHHHSGSGSGSGIEGRVRPLNSIVAVSQNMGIGKNGDLPWPPLRNEFKYFQR
MTTSSVEGKQNLVIMGRKTWFSIPEKNRPLKDRINIVLSRELKEPPRGHAHFLAKSLD
DALRLIEQPELASKVDMVWIVGGSSVYQEAMNQPGHLRLFVTRIMQEFESDTFFPEID
LGKYKLLPEYPGVLSEVQEEKGIKYKFEVYEKKD

N_{term}-mDHFR

MRGSHHHHHHSGSGSGSGIEGRVRPLNSIVAVSQNMGIGKNGDLPWPPLRNEFKYFQR
MTTSSVEGKQNLVIMGRKTWFSIPEKNRPLKDRINIVLSRELKEPPRGHAHFLAKSLD
DALRLIEQPELC

C_{term}-mDHFR (1)

GCASKVDMVWIVGGSSVYQEAMNQPGHLRLFVTRIMQEFESDTFFPEIDLGKYKLLP
EYPGVLSEVQEE KGIKYKFEVYEKKGSRSHHHHHH

C_{term}-mDHFR (2) (linker appears in bold)

GCGGSGGASKVDMVWIVGGSSVYQEAMNQPGHLRLFVTRIMQEFESDTFFPEIDLG
KYKLLPEYPGVLSEVQEEKGIKYKFEVYEKKGSRSHHHHHH

Reference List

- [1] J. Sambrook, D. Russel. *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, NY, Cold Spring Harbor Laboratory Press, **2001**.
- [2] G. P. Miller, D. C. Wahnnon, S. J. Benkovic. *Biochemistry* **2001**, 40(4), 867-875.
- [3] <http://www.expasy.org/tools/protparam.html>.